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Moderately lower temperatures greatly extend the lifespan of *Brachionus manjavacas* (Rotifera): Thermodynamics or gene regulation?

Rachel K. Johnston *, Terry W. Snell

School of Biology, Georgia Institute of Technology, Atlanta, GA 30332-0230, USA

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ABSTRACT

Environmental temperature greatly affects lifespan in a wide variety of animals, but the exact mechanisms underlying this effect are still largely unknown. A moderate temperature decrease from 22 °C to 16 °C extends the lifespan of the monogonont rotifer *Brachionus manjavacas* by up to 163%. Thermodynamic effects on metabolism contribute to this increase in longevity, but are not the only cause. When rotifers are exposed to 16 °C for four days and then transfered to 22 °C, they survive until day 13 at nearly identical rates as rotifers maintained at 16 °C continuously. This persistence of the higher survival for nine days after transfer to 22 °C suggests that low temperature exposure alters the expression of genes that affect the rate of aging. The relative persistence of the gene regulation effect suggests that it may play an even larger role in slowing aging than the thermodynamic effects. The life extending effects of these short-term low temperature treatments are largest when the exposure happens early in the life cycle, demonstrating the importance of early development. There is no advantage to lowering the temperature below 16 °C to 11° or 5 °C. Rotifers exposed to 16 °C also displayed increased resistance to heat, starvation, oxidative and osmotic stress. Reproductive rates at 16 °C were lower than those at 22 °C, but because they reproduce longer, there is no significant change in the lifetime fecundity of females. To investigate which genes contribute to these effects, the expression of specific temperature sensing genes was knocked down using RNAi. Of 12 genes tested, RNAi knockdown of four eliminated the survival enhancing effects of the four-day cold treatment: TRP7, forkhead box C, Y-box factor, and ribosomal protein S6. This demonstrates that active gene regulation is an important factor in temperature mediated life extension, and that these particular genes play an integral role in these pathways. As a thermoresponsive sensor, TRP7 may be responsible for triggering the signaling cascade contributing to temperature mediated life extension. The TRP genes may also provide especially promising candidates for targeted gene manipulations or pharmacological interventions capable of mimicking the effects of low temperature exposure. These results support recent theories of aging that claim rate of aging is determined by an actively regulated genetic mechanism rather than an accumulation of molecular damage.

1. Introduction

Many environmental and genetic factors interact to affect aging rate, and a deeper understanding of these mechanisms is integral to discovering new interventions to slow the rate of aging (Magalhães et al., 2012). Emerging evidence is shifting the traditional views of aging as an accumulation of molecular damage to an actively regulated signaling network (Gems and Partridge, 2013). Even small changes affecting the interconnected pathways can lead to large changes in longevity, especially when applied over the entire lifespan (Conti, 2008).

Temperature is a key environmental factor that greatly affects lifespan in all animals studied. There are many cases where modestly lower environmental temperatures greatly increase the lifespan of ectotherms. For example, a 5 °C decrease in water temperature extends lifespan of a small, South American fish (genus *Cyanolebias*) by nearly 100% (Rikke and Johnson, 2004). In another fish model, lowering environmental temperature from 25 °C to 22 °C caused not only a significant increase in mean and maximum lifespan, but also delayed the onset of aging markers such as diminished cognitive ability and lipofuscin accumulation (Valenzano et al., 2006). In lakes with thermal gradients, *Daphnia* that prefer the colder waters have lower metabolic rates and live several times the usual lifespan (Pietrzak et al., 2013). Because of the internal mechanisms for maintaining a constant temperature, these effects are harder to study in homeotherms (Carrillo and Flouris, 2011). However, transgenic mice with a 0.3 °C to 0.5 °C reduction in core body temperature had 12 to 20% longer lifespans than controls, regardless of diet (Conti et al., 2006).

Although the exact mechanism of temperature mediated life extension remains largely unknown, it is thought to be related to the same mechanisms by which caloric restriction (CR) extends lifespan. In

* Corresponding author at: School of Biology, 310 Ferst Dr, Institute of Technology, Atlanta, GA 30332-0230, USA.
E-mail address: rachel.johnston@biology.gatech.edu (R.K. Johnston).

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homeotherms, CR is associated with a lower core body temperature because it lowers metabolic rates (Conti, 2008). The magnitude of the body temperature response to CR varies with different genetic strains of mice (Rikke and Johnson, 2004). In the fish *Cynolebias*, CR combined with reduced environmental temperature produced an additive effect on longevity, extending lifespan by nearly 300% (Rikke and Johnson, 2004). Several models suggest overlapping mechanisms by which CR and body temperature affect longevity. These include genetic regulation, reductions in inflammatory response, decreased oxidative damage, increased metabolic efficiency, and hormone regulation (Carrillo and Flouris, 2011; Conti, 2008).

These regulatory pathways are likely highly interconnected and active gene regulation seems to play a major role in temperature mediated extension of longevity (Lee and Kenyon, 2009). It is also appreciated that passive thermodynamic effects contribute to the temperature effect, but how much remains to be quantified. In *C. elegans*, it was found that life extension by lower environmental temperatures is dependent on a thermosensitive genetic pathway, a TRP channel, a calcium sensitive PKC, SGK, and the DAF-16/FOXO transcription factor (Xiao et al., 2013). The DAF-16/FOXO pathway is linked to the nutrient sensing pathway, further connecting the mechanisms of temperature and CR. TRP channels are further known to play a role in external and internal environmental sensing and longevity regulation (Linford et al., 2011). Additionally, the expression of many genes is known to be regulated by changes in temperature. In zebrafish, a shift from 25 °C to 12 °C increased expression of ependymin, suggesting a role in cold acclimation (Tang et al., 1999). In the rotifer *Brachionus manjavacas*, a one hour 40 °C heat shock increased the expression of four heat shock proteins (HSPs) which play an essential role in thermotolerance and stress protection (Smith et al., 2012). In plants, a 15 min exposure to 4 °C increases the expression of CBF (cold-repeat binding factors), transcriptional factors that trigger a signaling cascade inducing freezing tolerance (Thomashow, 2010).

Members of the phylum Rotifera are a useful model for studying aging mechanisms (Snell, 2014). Rotifers are members of the understudied supra-phylum Lophotrochozoa. They have a short lifespan of about 12 days at 22 °C, which allows full life tables experiments to be performed in a short time (Snell et al., 2014a). RNAi can be used to effectively knockdown gene expression of selected genes, and biological effects can be observed to better understand the pathway or mechanism by which these genes are acting (Snell et al., 2014b). In this study, we use the rotifer *B. manjavacas* to explore the effects of different temperature treatments on longevity and identify genes important to temperature-mediated changes in lifespan. We hypothesized that exposure to lower temperatures would greatly extend rotifer lifespan, through both thermodynamic effects and gene regulation. Thermodynamic effects alone cannot account for the observed life extension. We also hypothesized that knockdown in the expression of temperature sensing genes can lead to large changes in longevity and reproduction.

2. Materials and methods

2.1. Rotifer culture

All experiments were performed using the rotifer species *B. manjavacas*. This strain was originally collected from the Azov Sea in Russia, and has been continuously propagated in the laboratory since 1983, with resting eggs being periodically collected, dried, and stored (Rico-Martinez and Snell, 1995). Before each experiment, *B. majavacas* resting eggs were hatched in 25 mL, 15 ppt artificial sea water (ASW, Instant Ocean) under constant fluorescent illumination (2000 lx) at 25 °C. Under these conditions, hatching begins after 18–20 h, resulting in a uniform cohort of neonates. For particular treatments, resting eggs were hatched at 16 °C. At this temperature, hatching was delayed until 48 h. Hatchlings were fed the green alga *Tetraselmis suecica* cultured in modified F medium (Guillard, 1983) in a 560 mL chemostat with 25% daily medium replacement, at 25 °C and constant fluorescent illumination of 2000 lx. For experiments investigating the generality of cold response, resting eggs were harvested from three additional rotifer strains; *B. manjavacas* from Pettaquamscutt, Rhode Island (Suatoni et al., 2006), *B. plicatilis* from China (Snell, 2014), and *B. rotundiformis* from Hawaii (Rico-Martinez and Snell, 1995). These strains were cultured under the same conditions as *B. manjavacas* (Russian).

2.2. Experimental design and treatments

Full cohort life table experiments were conducted with 120 newly hatched female *B. manjavacas* per treatment. Animals were kept in 24-well plates with each well containing 5 females in 1 mL of Medium. Medium contained 6×10^5 *T. suecica* cells/mL in 15 ppt ASW and 20 μM 5-fluoro-2-deoxyuridine (FDU), added to prevent the hatching of asexual eggs (Snell et al., 2014b), with the exception of *B. rotundiformis* medium which contained only 8 μM FDU. Plates were checked daily and mortality was recorded until all animals were dead. All animals were transferred to new plates with fresh medium every 8 to 16 days as needed to replenish food supply.

Reproductive life table experiments were performed in a similar fashion, with a few modifications. Single females were kept in each well of 24-well plates in 1 mL of medium, for a total of 24 females per treatment. Medium contained 2×10^5 *T. suecica* cells/mL in 15 ppt ASW, and no FDU was added to allow normal egg hatching. Offspring were produced parthenogenetically and were counted and removed daily. The original maternal females were transferred to new plates every 6 to 12 days, depending on food supply.

All plates were kept in the dark at the appropriate temperature in Percival I-41VL incubators. Standard control temperature was 22 °C, with low temperature treatments of 20, 18, 16, 11 and 5 °C. 22 °C is used as standard incubation temperature because at this temperature, reproductive experiments can be scored accurately at 24 h intervals. The plates were removed from the incubators once a day and scored at room temperature (22 °C). Care was taken to minimize the time the plates were removed from the appropriate temperature treatment.

2.3. Stressor challenge experiments

Five stressor challenge experiments were conducted to test the effects of low temperature exposure on resistance to starvation, oxidative stress, UV exposure, osmotic shock, and heat shock. All animals were kept in 6-well plates with each well containing several dozen animals in 5 mL medium. Medium consisted of 6×10^5 *T. suecica* cells/mL in 15 ppt ASW with 20 μM FDU. Plates were stored in the dark at 16 °C for either 0, 1, 2 or 4 days before being transferred to 22 °C. On the fourth day, animals were rinsed with 15 ppt ASW to clear away algae, and challenged with one of five stress treatments.

Rotifers in the starvation treatment were transferred to 15 ppt ASW and deprived of food for 72 h. Osmotic shocked animals were transferred from 15 ppt to 60 ppt ASW in a small petri dish for 1 h, then returned to 15 ppt. Heat shocked animals were transferred to a petri dish where they were incubated at 40 °C for 1 h then returned to 22 °C. UV stressed animals were transferred into a small petri dish in 5 mL 15 ppt ASW and exposed to UV-B radiation for 20 min. The dish was 25 cm from an 8 W light source (UVP, model UVM-28EI) with an intensity peak at 302 nm. After undergoing these stress treatments, these animals were transferred to 24-well plates with a total of 120 animals per treatment. Each well consisted of 10 animals and 1 mL of 15 ppt ASW with 20 μM FDU. In the oxidative stress treatment, the medium in the plates also contained 0.1 μM juglone. These stress treatments were chosen to produce considerable, but not 100%, mortality in the control. All plates were stored in the dark at 22 °C, and scored for survival after 72 h. Survival is reported as the average percentage surviving in each treatment.

2.4. Synthesizing and purifying dsRNA

Genes putatively related to temperature sensing pathways were derived from the *B. manjavacas* Transcriptome Shotgun Assembly Project. These sequences are available at DDBJ/EMBL/GenBank under the accession GARS01000000. Twelve genes were selected based on sequence similarity to genes identified by Xiao et al. (2013) and other temperature sensing pathways. Primers were designed to amplify a 500 bp region from each rotifer gene, and DNA product was sequenced using Eurofins Genomics SimpleSeq to ensure proper amplification of the genes of interest. Primers and accession numbers for all genes can be found in Table 1.

To collect DNA for dsRNA synthesis, 0.5 μM of forward and reverse primers with a T7-motif (TAATACGACTCACTATAGG) were used with Go-Taq DNA polymerase (Promega), 1 mM $MgCl_2$, 100 mM dNTP (Promega), 10× buffer, and 40 ng of genomic *B. manjavacas* DNA. The resulting PCR products were purified using a QIAquick PCR purification kit (Qiagen). dsRNA was synthesized from the T7 tagged DNA and purified using a MEGAscript® RNAi kit (Invitrogen). Purified dsRNA was quantified using a NanoDrop 1000 (Thermo Scientific) and visualized on a 1% agarose gel to verify band size.

To serve as a control for mortality or changes in expression due to exposure to transfection reagents, a scrambled sequence of the rotifer TOR gene was synthesized by the company IDT and used in place of genomic DNA in the initial PCR, as described by Snell and Johnston (2014). The scramble sequence is not found in the known *B. manjavacas* genome, and was used as a control in all RNAi experiments.

2.5. RNAi knockdown experiments

Four types of RNAi experiments were performed to screen for biological effects of gene expression knockdown. The biological effects tested were ability of resting eggs to hatch, female survival, reproduction, and cold sensitivity. For all experiments, a transfection solution was prepared with 47 μL PBS, 1 μL FuGene6 (Promega), and 2 μL (200 ng) dsRNA and incubated at room temperature for 20 min (modified from Snell et al., 2014b). For hatching, survival, and cold sensitivity tests, *B. manjavacas* neonates hatching from resting eggs were collected within 2 h of their births, and transferred to 500 μL 15 ppt ASW in a 24-well plate. Roughly 100 animals were transferred to each well. 50 μL of transfection solution was added to each well, and the plate was incubated at room temperature for 4 h before animals were transferred to each experimental treatment.

For the survival experiments, animals were transferred to 24-well plates with 1 mL 6×10^5 *T. suecica* cells/mL, 20 μM FDU, and 5 females in each well. One full plate was used for each treatment, and plates were incubated at 28 °C in the dark for eight days. On days 2, 4, and 6, plates were treated with 5 μL additional FDU (1 mg/mL) to prevent asexual egg hatching, and on day 6 plates were fed 100 μL of 6×10^6 *T. suecica* cells/mL per well. After 8 days, the number of living animals was counted and survival was recorded as average percent surviving in each treatment.

In the reproductive experiments, animals were transferred from the transfection solution into 24-well plates with 1 mL 2×10^5 *T. suecica* cells/mL and 1 female in each well. One full plate was used for each treatment, and the plates were incubated in the dark at 25 °C for 72 h. Offspring were counted and removed daily, and population growth rate r was calculated at 72 h.

To screen for changes in lifespan due to cold sensitivity, animals were transferred into 24-well plates with 1 mL 6×10^5 *T. suecica* cells/mL, 20 μM FDU, and 5 females in each well. For each treatment, 3 full plates were prepared. One of these plates was incubated at 22 °C continuously, another was incubated at 16 °C continuously, and the third was incubated at 16 °C for the first four days and then transferred to 22 °C. On days 4, 8, and 12, each well was treated with 5 μL additional FDU (1 mg/mL), and on day 8 each well was fed 100 μL 6×10^6 *T. suecica* cells/mL. Survival was recorded daily until day 15, and the data was used to generate right-censored survival curves.

To test the effects of RNAi knockdown on hatching, diapausing *B. manjavacas* embryos (resting eggs) were decapsulated using the protocol of Snell et al. (2010), and then transferred to 24-well plates with 500 μL 15 ppt ASW, 25 μL transfection solution, and 20 resting eggs in each well. Six replicate wells were used for each treatment. Plates were incubated at 25 °C under constant fluorescent illumination (2000 lx). Number of eggs hatched in each well was recorded after 48 h.

2.6. Statistics

Survival curves from full life tables and cold sensitivity screens were evaluated using the JMP Pro 11 (SAS Institute) reliability and survival analysis with a Wilcoxon's test to compare control and treatments. Stress challenge, hatching, 8 day survival, and reproductive screens were compared by ANOVA with Dunnett's test to compare treatments to control.

3. Results

Small changes in environmental temperature lead to large changes in rotifer lifespan (Fig. 1). A 2 °C decrease from 22 °C to 20 °C extended lifespan by 39%. A further 2 °C decrease to 18 °C extended lifespan by 83%, and a 6 °C decrease from 22 °C to 16 °C extended lifespan by 115% compared to the 22 °C control. Similarly, a 6 °C increase in temperature to 28 °C decreased lifespan by 52%. All of these temperature induced changes in survival are significantly different from the 22 °C control ($P < 0.0001$) by a Wilcoxon test.

A second experiment examined the effects of hatching the resting eggs at different temperatures on adult survival and lifespan (Fig. 2). Hatching resting eggs at 16 °C and incubating the newborns at 22 °C produced lifespans not significantly different from control animals hatched at 25 °C and incubated at 22 °C. Animals hatched at 25 °C and

Table 1
List of putative temperature sensing genes and the primer sequences used in PCR. T7 primers contain an additional T7 adapter at the 5′ end. All genes can be found within the *Brachionus manjavacas* transcriptome shotgun assembly (GARS01000000) using the accession numbers listed.

Gene	Abreviation	GB Accession	Related gene/pathway	Forward	Reverse
Protein kinase C 1	PKC1	GARS01002868.1	SGK (HUMAN)	GGCTGGTTCAAACTGTTGGT	ATCGCCACCGTTGATGTATT
Protein kinase C 2	PKC2	GARS01002161.1	PKC (Human)	TTGAAGAATTGCGTCACAGG	CGCACATTGTAGGTCTTGGA
Ribosomal protein S6 polypeptide 2	S6P	GARS01003002.1	SGK (Human)	GCGATGACGACAATATCACG	TCCAACTGCATGAACAGCTC
Ribosomal protein S6 kinase alpha-1	S6Ka	GARS01002535.1	SGK (Human)	CAAGCCGACTCCATCTCATT	CAGGCTTTAGATCGCGGTAA
Transient receptor potential cation subfamily member 1	TRP1	GARS01002238.1	TRP (Human)	GCTGGCCAGACAAATACTCG	ATACGCCGAAAGCAAAGAAA
Transient receptor potential cation subfamily member 7	TRP7	GARS01012197.1	TRP (C elegans)	CAATGTTTTGGTCCTTATTTGG	CCAAATGGAATTGATCCGATA
Forkhead transcription factor n1 4 transcript variant 1	FhTF	GARS01002302.1	Daf 16-g (C elegans)	CCCAAGTGGCTATGAGAAGC	TTAGCTTGCGTTGACAGGTG
Forkhead box C-like protein	FhBC	GARS01006072.1	Daf 16-a (C elegans)	AACGACTGCTTCTGCAAGGT	GACCGAAAACGACGAGTAGG
Fork-head box j2 3 transcription Factor	FhBJ	GARS01005585.1	Daf 16-h (C elegans)	CATCAATGGAATGGTTGTCG	AACCCTTTCCAGGGTCATCT
Y-box factor	YBoxF	GARS01001540.1	Temperature Sensitivity	TGTCGGATACCGCAGTACAA	TTTGGTGTCGTCACGGTTTA
Y box binding protein 1	YBoxBP	GARS01002878.1	Temperature Sensitivity	TTGAACCAGTGTCCCAACAA	GGCTGTCTTGGTGGTCTGTT
Mammalian ependymin-related protein	MERP	GARS01009784.1	MERP (D rerio)	ACCATGCACATCTCCTCCTC	CGATTCCTGGAACAATGTCG

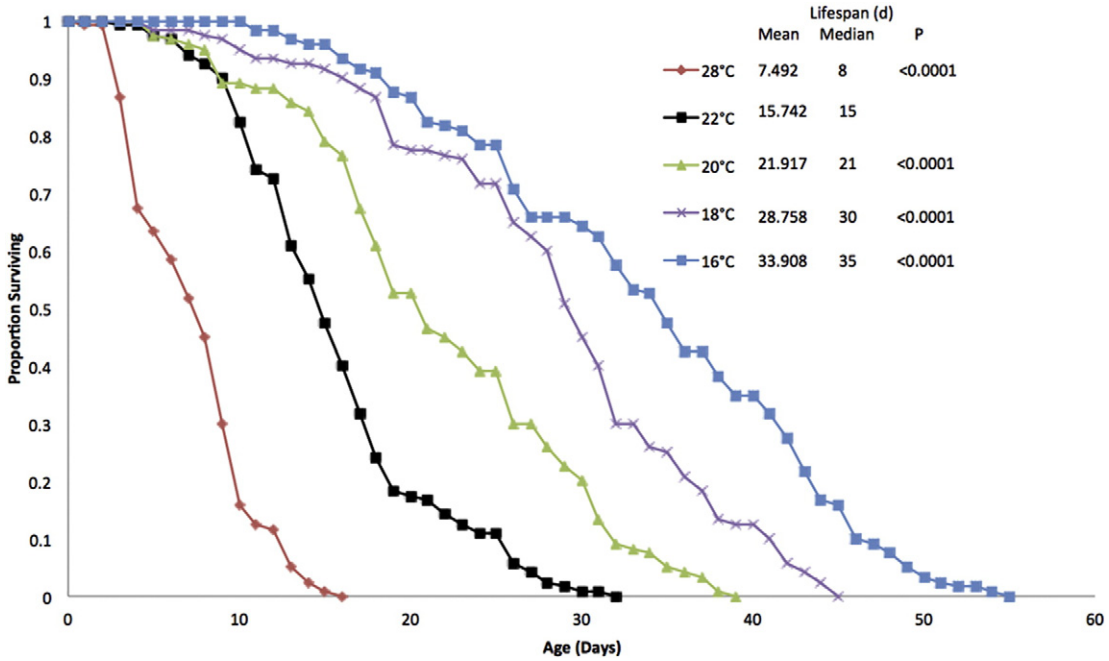

Fig. 1. Survival curves for *B. manjavacas* incubated continuously at 5 different temperatures. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days, and P represents the Wilcoxon test probability that the treatment curves are similar to the 22 °C control.

incubated at 16 °C produced significant life extension of 63% as compared to control. However, animals that were hatched at 16 °C and maintained at 16 °C experienced an 86% longer lifespan, significantly greater than rotifers hatched at 25 °C and transferred to 16 °C.

A third life table experiment was performed to explore the effects of 16 °C exposure at various life stages (Fig. 3). Continuous 16 °C exposure extended lifespan by 163%. Exposing rotifers to 16 °C for only their first four days significantly extended lifespan by 31%. Even though exposure to 16 °C only lasted until day four, the survival curve closely followed that of the 16 °C continuous treatment until day 13. Exposing the animals to 16 °C from days 4 to 8 also extended lifespan by 16%, but was not nearly as effective at extending lifespan as the same exposure in the early age classes. Exposure to 16 °C from days 8 to 12 provided no significant life extension.

A fourth life table experiment was performed to investigate whether additional exposure to 16 °C could extend the protective effect of the initial four day 16 °C exposure (Fig. 4). In this experiment, 16 °C exposure for the first four days did not significantly increase median lifespan, but survival was essentially the same as the 16 °C continuous treatment until day 11. However, a 16 °C exposure for the first four days, followed by alternating four days at 22 °C and then four days at 16 °C for the remainder of the lifespan significantly extended lifespan by 24%, and the survival curve followed that of the 16 °C continuous treatment until day 16.

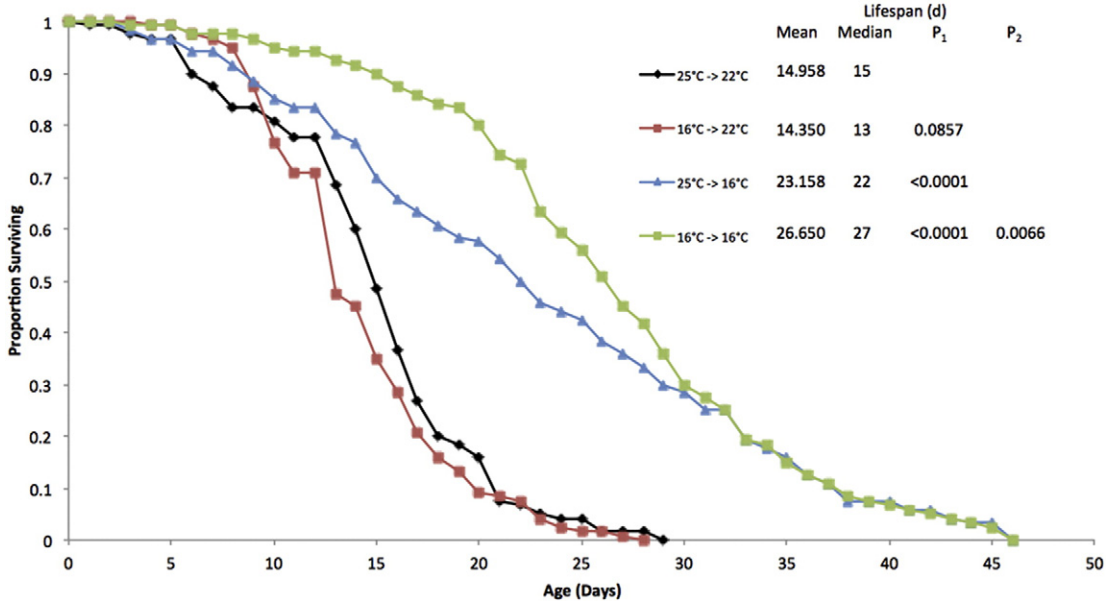

Fig. 2. Survival curves for *B. manjavacas* hatched and incubated at different temperatures. The first number of the treatment refers to the temperature resting eggs were hatched in and the second refers to the incubation temperature after hatching. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days. P_1 represents the Wilcoxon test probability that the treatment curves are similar to the 25 °C– > 22 °C control, and P_2 represents the Wilcoxon test probability that the curve is similar to the 25 °C– > 16 °C treatment.

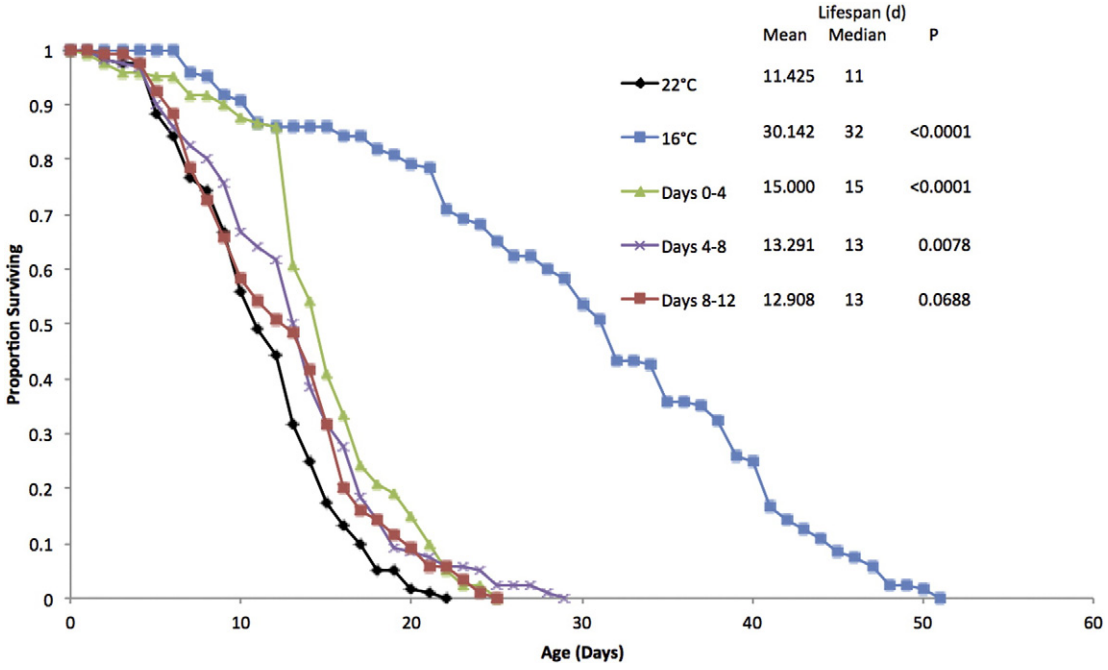

Fig. 3. Survival curves for *B. manjavacas* incubated at different temperatures during different stages of the lifespan. The first two treatments remained at a single continuous temperature. The other three treatments were incubated at 16 °C for the days labeled and 22 °C for all other days. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days, and P represents the Wilcoxon test probability that the treatment curves are similar to the 22 °C continuous control.

The effects of a range of early life cold treatments were examined in another life table (Fig. 5). In this experiment, a four day exposure to 16 °C extended lifespan by 22%. A four day exposure to 11 °C similarly extended lifespan by 26%, but provided no significant additional benefit beyond exposure to 16 °C. A four day exposure to 5 °C on the other hand, provided no life extension, and the survival curve displayed an unusually large number of deaths in the early age classes. The rotifers exposed to 5 °C were also an average of 42% smaller than those incubated at 22 °C, and in many of them, the pseudocoelom was dark green, suggesting that the gut ruptured allowing algae to escape into the pseudocoelom.

The 22 °C continuous control treatments in these five independent life table experiments displayed small amounts of experimental variation (Table 3). The average lifespan of the control animals was 14.2 days with a 12.4% coefficient of variation. The average maximum lifespan of the longest-lived 10% of animals was 23.7 with a 15.1% coefficient of variation.

A reproductive life table was performed to observe the effect of a 16 °C temperature on lifespan and reproduction. In the absence of FDU, the 16 °C treatment extended lifespan by 122% as compared to the 22 °C control (Fig. 6a). The reproductive curves of the two

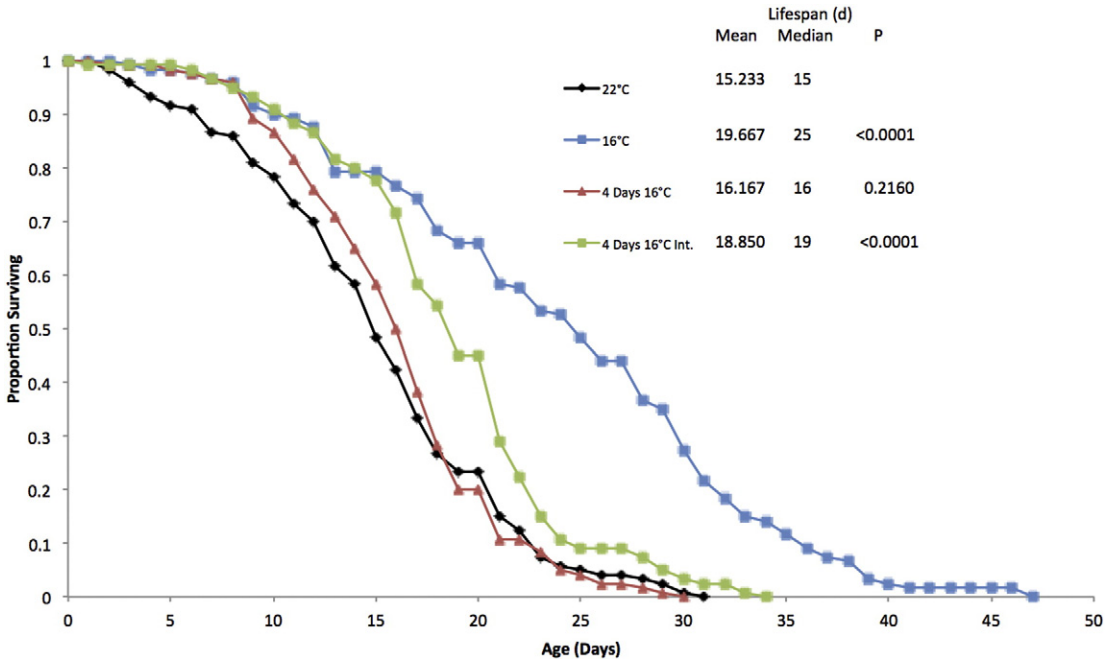

Fig. 4. Survival curves for *B. manjavacas* incubated at different temperature treatments. 4 Days 16 °C was exposed to 16 °C only for the first 4 days of life, while the Int. treatment was exposed for 4 days intermittently throughout the lifespan. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days, and P represents the Wilcoxon test probability that the treatment curves are similar to the 22 °C control.

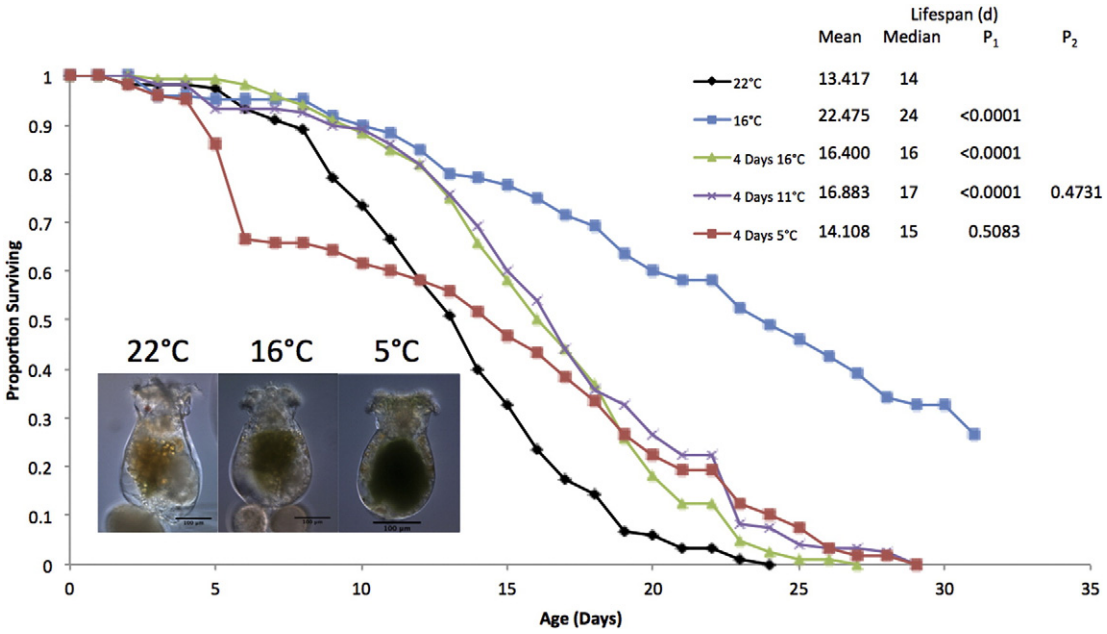

Fig. 5. Survival curves for *B. manjavacas* incubated at different temperatures for the first four days of the lifespan. All four day treatments were transferred to 22 °C on day 5. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days. P_1 represents the Wilcoxon test probability that the treatment curves are similar to the 22 °C control, and P_2 represents the Wilcoxon test probability that the curve is different from the 4 days at 16 °C treatment. Photos show 5 day old animals from different temperatures.

treatments were very different, with the 22 °C treatment peaking at nearly 7 offspring per female per day and earlier (day 4), whereas the 16 °C treatment produced fewer offspring (peak: 2.5 offspring per female per day, days 6–8) for a longer period of time (Fig. 6b). Nonetheless, the mean total offspring produced by a female over her lifespan (R_0) was not significantly different between the two temperatures. The average proportions of the life span spent in pre-reproductive, reproductive, and post-reproductive stages also did not differ significantly between treatments.

Another effect of exposure to 16 °C was to provide protection from different stressors, as demonstrated in five stress challenge experiments (Fig. 7). Four days of continuous 16 °C exposure provided significant protection from all five stressors as compared to animals incubated at 22 °C. One or two days of 16 °C exposure followed by 22 °C incubation until the stress tests on day 4 also provided significant protection from oxidative, osmotic, and heat stress. Two days of 16 °C exposure provided protection from starvation, but only one day exposure did not. Neither one nor two days of 16 °C treatment was enough to provide protection from UV light.

RNAi was used to explore the importance of potential temperature sensitive genes in mediating these life extension effects. Twelve rotifer genes were chosen based on sequence similarity to genes known to be involved in temperature sensing pathways in *C. elegans* (Table 1). A series of screens were used to assess the different types of biological responses to RNAi knockdown of these genes (Table 2). Only PKC1 knockdown had any significant effect on resting egg hatching, decreasing the mean

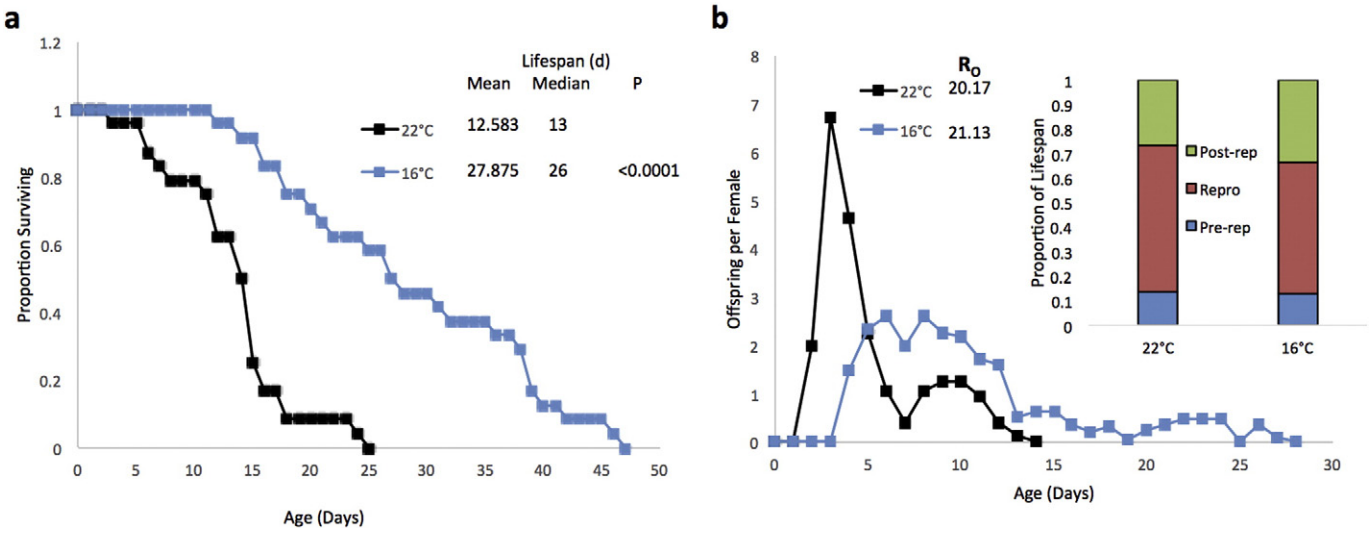

Fig. 6. a) Survival curves for *B. manjavacas* incubated continuously at 2 different temperatures. b) Reproductive curves for *B. manjavacas* incubated at 2 different temperatures. Proportion surviving represents the fraction of an initial cohort of 24 females surviving to each age. Mean and median lifespan are reported in days, and P represents the Wilcoxon test probability that the treatment curves are similar to the 22 °C control. Offspring per female refers to the average number of offspring produced each day by a cohort of 24 females. R_0 is the mean total offspring produced by a female over the lifetime. Pre-rep refers to the average proportion of the lifespan a female spends in the pre-reproductive stage. Repro is the proportion spend reproducing, and Post-rep is the proportion spent in the post-reproductive stage.

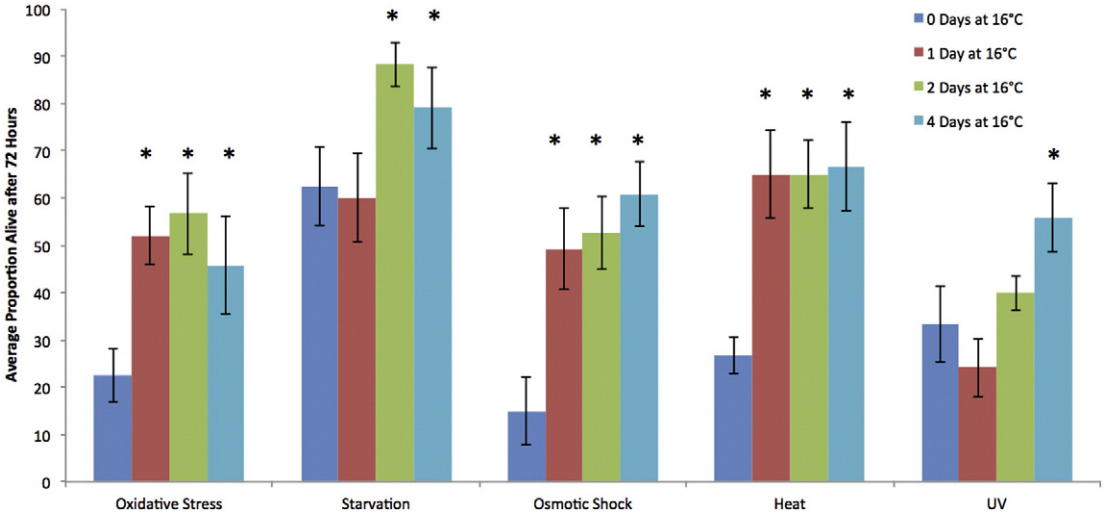

Fig. 7. The effects of different stressors on *B. manjavacas* survival. All treatments were incubated at 22 °C when not in the 16 °C treatment specified, and all stress challenges were applied on day 4. Oxidative stress exposed animals to 0.1 μM juglone for 72 h. Animals in the starvation test were removed from all food for 72 h. Osmotic shock exposed animals to 60 ppt ASW for 1 h. Heat shock exposed animals to 40 °C for 1 h. UV treatment animals were exposed to UV light for 20 min. Variance is represented by the standard error bars on each column. Asterisks denote a P-value of <0.05 calculated from an ANOVA with Dunnett's test comparing the treatments to the control for each stressor.

number of eggs hatched after 48 h by 73%. RNAi knockdown of PKC1, S6P, TRP7 and YBoxBP genes all significantly decreased average number of offspring produced by asexual mothers after 72 h. Most interestingly, S6Kα, TRP7, and FhBC knockdown increased mean survival after eight days at 28 °C by 36%, 23% and 29%, respectively.

To assess the role of these genes in rotifer temperature sensing more specifically, screens were conducted to compare the survival of RNAi treated animals at 22 °C continuous, 16 °C continuous, and a four day 16 °C exposure before being transferred to 22 °C (Fig. 8). Control animals were treated with a scrambled dsRNA from the rotifer TOR gene. A Wilcoxon's test showed that both the four day and continuous 16 °C treatments have very similar survival, and that both treatments survived significantly better than the 22 °C treatment. However, four of the RNAi knockdown treatments markedly altered the survival curves. When rotifers were exposed to dsRNA of TRP7, FhBC, YBoxF, and S6P genes, the four day 16 °C exposure curve showed significantly lower survival than the 16 °C continuous, and was much more similar to 22 °C continuous exposure. This shift in the effect of a 16 °C exposure on rotifer survival demonstrates that these four genes likely have a key role in sensing and responding to low temperatures by rotifers.

To investigate the generality of this life extension response by animals treated at 16 °C for four days, similar screens were performed on four different *Brachionus* strains (Fig. 9). In *B. manjavacas* (Russian) and *B. manjvacas* (Petta), both the four day treatment and continuous 16 °C treatment survived significantly better than the 22 °C control. *B. plicatilis* (China), followed a similar pattern, but the persistent survival effect of the four day treatment was not as strong. In *B. rotundiformis* (Hawaii), the response to cold treatment was much different. The animals at 22 °C continuously actually survived significantly better than those cultured at 16 °C continuously. Those treated at 16 °C for four days survived significantly better than those at 16 °C continuously, and did not differ significantly from the 22 °C treatment.

Table 2
The effects of RNAi mediated knockdown of temperature related genes on *B. manjavacas*. Hatching refers to the average number of resting eggs hatched after 48 h. Reproduction refers to average number of offspring per female at 72 h. Survival refers to the average proportion of animals surviving after 8 days at 28 °C. NS denotes no significant difference from the scramble treated control ($P > 0.05$) as calculated by ANOVA with Dunnett's test. Percentages refer to significant increase or decrease as compared to control.

Gene	Hatching	Reproduction	Survival
PKC1	↓72.5%	↓15.8%	NS
PKC2	NS	NS	NS
S6P	NS	↓16.1%	NS
S6Ka	NS	NS	↑36.2%
TRP1	NS	NS	NS
TRP7	NS	↓17.2%	↑23.2%
FhTF	NS	NS	NS
FhBC	NS	NS	↑29.0%
FhBJ	NS	NS	NS
YBoxF	Ns	NS	NS
YBoxBP	NS	↓21.9%	NS
MERP	NS	NS	NS

4. Discussion

The initial life table experiment demonstrated that even modest changes in temperature (9–27%) could greatly extend rotifer lifespan (Fig. 1). Each 2 °C reduction in temperature resulted in roughly 40% lifespan extension. A 6 °C temperature difference from 22 °C to 16 °C provided the greatest lifespan increase up to 163%. Although a 6 °C difference in core body temperature would be sizeable in a homeotherm, it lies well within the thermal tolerance range for a *Brachionus* rotifer (Walker, 1981).

It is well documented that across a variety of species including microbes, ectotherms, and endotherms, metabolic rate decreases with decreasing temperature by reducing the rates of biochemical reactions (Gillooly et al., 2001). In tropical plants, temperatures below 15 °C disrupt vital physiological processes including photosynthesis and respiration, slowing and in some cases completely inactivating metabolism (Lutkatkin et al., 2012). Insects are able to rapidly acclimate to cold

Table 3
Data on the sample size, mean lifespan, and lifespan of the longest-lived 10% of individuals from all control treatments. Each experiment represents an independent cohort followed for the entire lifespan. Control treatments were incubated continuously at 22 °C with 6×10^5 cells/mL T. suecica and 20 μM FDU.

Experiment	n	Mean	Max (10%)
1	120	15.7	27.8
2	120	15.0	24.3
3	120	11.4	19.3
4	120	15.2	26.3
5	120	13.4	21.0
Mean		14.2	23.7
Standard deviation		1.8	3.6
Coefficient of variation		12.4	15.1

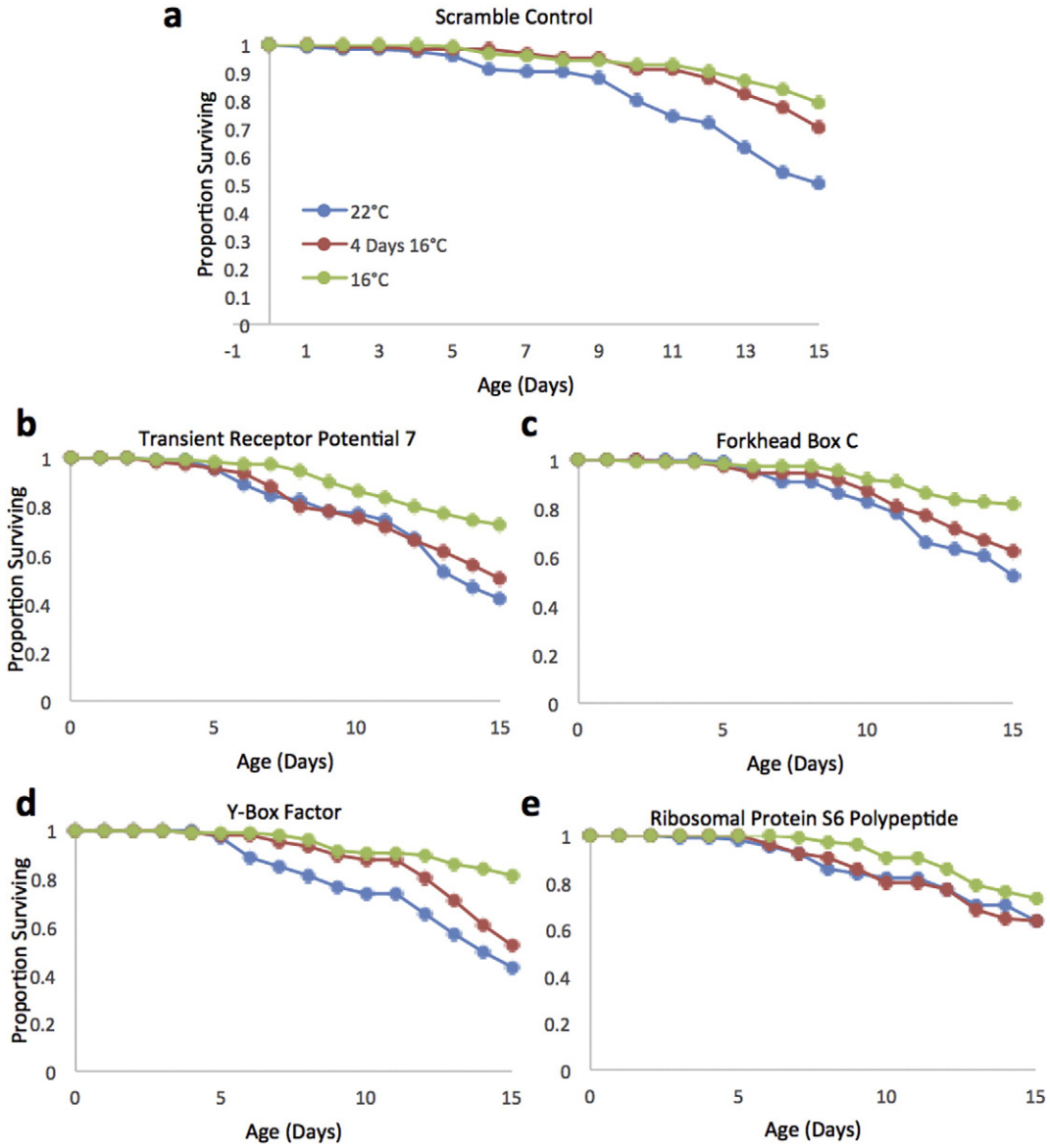

Fig. 8. Survival curves for RNAi treated *B. manjavacas* incubated at 3 different temperatures. a) Typical curves for the scramble treated control animals at each temperature. b–e) Survival curves for expression knockdown treatments where the persistent survival effect of a 4 day 16 °C exposure is eliminated. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age.

temperatures through a variety of physiological mechanisms (Teets and Denlinger, 2013). These responses include accumulation of cryoprotectants such as glycerol, a metabolic shift towards glycolysis, increases in membrane phospholipid unsaturation, and changes in calcium levels (Lee and Denlinger, 1991; Teets and Denlinger, 2013; Murray et al., 2007). However, these responses alone are not likely sufficient to explain the life extension effects found in our experiments.

A key finding from this study is the persistence of higher survival after removal from 16 °C. Survival rates for the four day 16 °C treatment matched that of continuous 16 °C exposure until day 12. This demonstrates that the mechanism of low temperature life extension cannot solely be a passive thermodynamic effect. It is quite likely that low temperature exposure modulated the expression of specific genes, thereby slowing aging processes. Clearly, identifying these genes and how they impact aging processes is a high priority in future work. Based on the duration of this persistent survival effect, and our findings that knockdown of specific genes can completely eliminate this effect, it is possible that genetic regulation elicited by changes in temperature plays an even larger role in temperature mediated life extension than passive thermodynamic effects. However, quantifying the relative magnitude of each mechanism requires additional research.

Although complete enumeration of the genetic pathways responsible requires further investigation, the life extending responses to lowered temperature are likely caused by a complex signaling cascade regulated by a sensory receptor. The mechanism of temperature sensation in plants remains incompletely understood, but within only 15 min of a 4 °C exposure, CBF transcription factors are induced. These transcription factors signal a large cascade of other genes involved in cold acclimation and stress resistance (Thomashow, 2010; Miura and Furumoto, 2013). In *C. elegans*, temperature mediated life extension was found to be dependent on the expression of the TRP channel TRPA-1 (Xiao et al., 2013). The TRP superfamily is comprised of a large number of cation channels with a variety of selective activation mechanisms. Many of the TRPs are thermosensory receptors that respond to a wide range of temperatures to trigger signaling cascades that regulate body temperature and avoid tissue damage (Flockerzi and Nilius, 2007). TRP thermoreceptors likely control this temperature mediated signaling cascade in rotifers as well. Because of the linkage between temperature, aging, and metabolism, it is likely that part of this cascade involves nutrient sensing pathways like TOR as well.

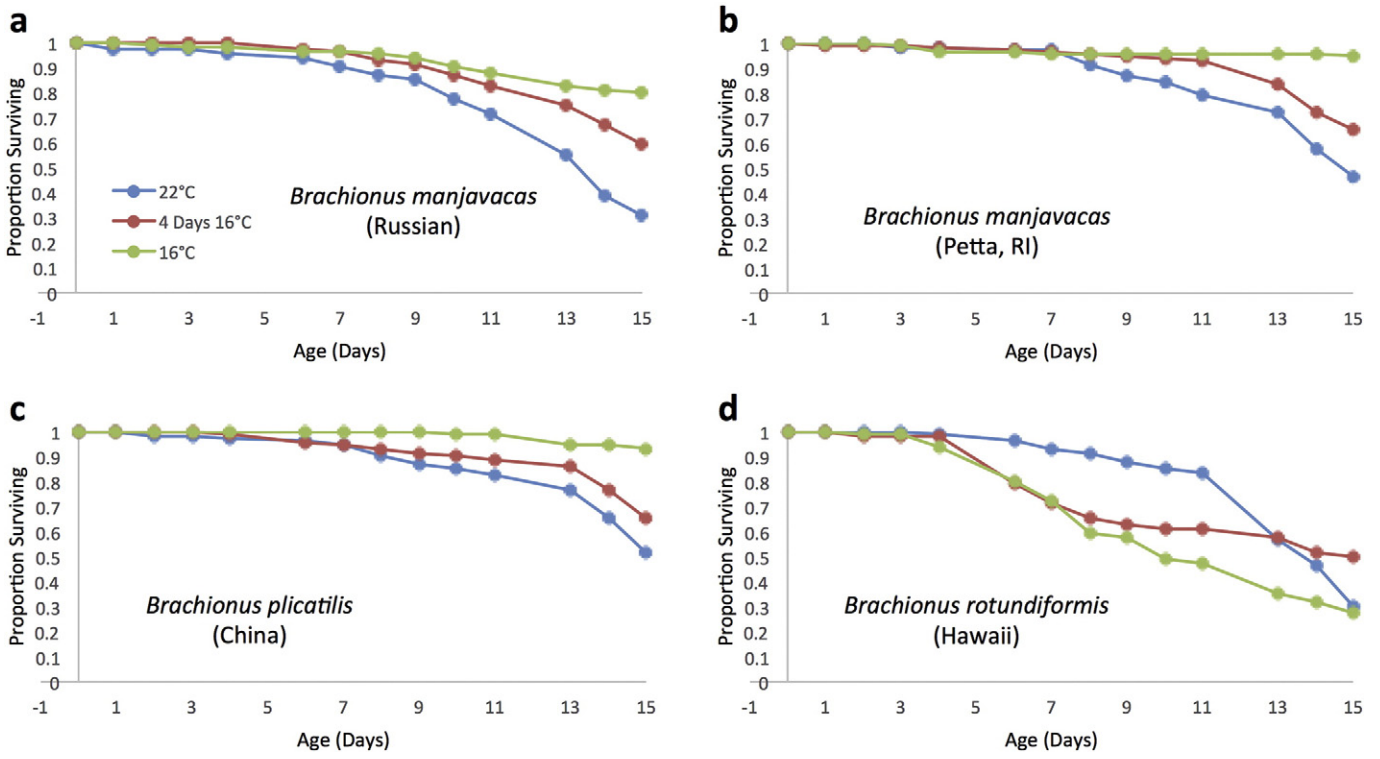

Fig. 9. Survival curves for *Brachionus* rotifers incubated at 3 different temperature treatments. a) Typical curves for *B. manjavacas* (Russian) at each temperature. b–d) Survival curves for other *Brachionus* strains and species. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age.

A series of RNAi experiments was used to further explore which genes may play an integral role in the thermosensory life extension of rotifers. The genes investigated in these experiments were all derived from the *B. manjavacas* transcriptome based on sequence similarity to genes found in temperature sensing pathways from other animals (Table 1). The high similarity between these genes suggests that they have been highly conserved evolutionarily and likely play an important role in metabolism. Of the 12 genes knocked down, only three (TRP7, FhBC, and S6Ka) caused a significant change in survival, and these effects were not nearly as large as the effects produced by a continuous 16 °C exposure (Table 2). Changing the expression of only one gene at a time was not sufficient to mimic the effects of a continuous low temperature treatment, further supporting the idea that multiple pathways are involved in temperature mediated life extension.

The persistent effect of a four day 16 °C treatment on survival was effectively eliminated in four of the gene knockdown treatments; TRP7, FhBC, YBoxF, and S6P. This emphasizes the likely role of these genes in temperature mediated life extension. FhBC and S6P are related to the DAF-16 and SGK genes, respectively. DAF-16 is a key regulator of both lifespan and stress resistance, and SGK is an upstream regulator of DAF-16 (Riedel et al., 2013; Xiao et al., 2013). TRP7 is a member of the TRP channel family, which are ancient, multifunctional thermosensory channels that can respond to both extracellular or intercellular activators (Linford et al., 2011). TRP7 specifically is related to the *C. elegans* TRPA-1 which detects environmental temperature drops to regulate a signaling cascade (Lee and Kenyon, 2009; Xiao et al., 2013). Finding ways to artificially manipulate sensory signaling through pharmacological or gene treatments could be a key to capturing the benefits of low temperature treatments on longevity without the need for prolonged low temperature exposure (Linford et al., 2011). The TRP channels provide excellent pharmacological targets for further research because there are several drugs that are already known to bind to mammalian thermosensory TRPs (Flockerzi and Nilius, 2007). Further experimentation is necessary to understand these types of gene–environment interactions and to discover new aging interventions (Magalhães et al., 2012).

The stress challenge experiments demonstrated that a 16 °C temperature treatment provides protection to a variety of stressor types (Fig. 7). This further supports the idea that temperature mediated longevity is controlled by a variety of interconnected pathways. Many life-extending interventions are associated with increased stress resistance, so it is not surprising that continuous low temperature exposure provides protection (Johnson et al., 1996). However, even a one-day exposure to 16 °C provides significant protection against oxidative, osmotic, and heat stress four days later. This provides additional evidence that temperature effects may be caused by the induction of specific genes. There is already considerable support for the idea that stress resistance has a genetic basis and that resistance can arise as an adaptation to abiotic environmental factors (Dragosits et al., 2013).

Lower temperatures may also confer resistance to stressors through the production of molecular chaperones. Heat shock proteins (HSPs) are well characterized, highly conserved molecular chaperones up-regulated after exposure to high temperatures (Fabbri et al., 2008). They stabilize proteins and increase thermotolerance in many animals (Smith et al., 2012). There is evidence that under cold stress, plants exhibit both post-transcriptional and post-translational regulation. RNA binding proteins prevent RNA from becoming over-stabilized in misfolded conformations at low temperatures, and cold stress affects an ubiquitin E3 ligase pathway to regulate the proteasome (Miura and Furumoto, 2013). In addition, cold stress has been shown to up-regulate HSP70 in *Drosophila* (Sinclair et al., 2007). Furthermore, the activation of thermosensitive TRPs in mammalian skin cells has been found to induce the expression of HSPs (Hsu and Yoshioka, 2015).

In the reproductive assay, it was found that while an incubation temperature of 16 °C increased lifespan by 122% as compared to a 22 °C control group, the lifetime fecundity of an individual female was unchanged (Fig. 6). In addition, the proportion of the lifespan spent in the reproductive phase was unchanged by temperature. This demonstrates that lower temperatures increase health-span as well as lifespan. Females at 16 °C produced fewer offspring per day, but remained reproductive

longer. This is consistent with previous research that found that in *B. plicatilis*, increasing temperature increased intrinsic rate of increase, but the effect of temperature on fecundity was negligible as long as the temperature was within the normal range for natural habitats (Miracle and Serra, 1989). However, it was also discovered that the slope of the relationship between temperature and reproduction was dependent on the genotype of the strain, and there were distinct differences between warm- and cold-adapted genotypes (Miracle and Serra, 1989).

This same phenomenon has been observed between rotifer sibling species in both natural populations and laboratory settings. In Spain, it was found that populations of *B. plicatilis* and two of *B. rotundiformis* were adapted to different temperature and salinity preferences. As such, the three genotypes demonstrated different responses in sexual reproduction to different temperature treatments (Gómez et al., 1997; Serra et al., 1998). We have observed that the lifespans of three closely related rotifer species (*B. manjavacas*, *B. plicatilis*, and *B. calyciflorus*) each respond differently to 16 °C. While the lifespan of *B. manjavacas* increases up to 163% as compared to animals at 22 °C, *B. plicatilis* lifespan increased by 84% and *B. calyciflorus* lifespan was increased by only 32% (unpublished observations).

Furthermore, different strains and species respond differently to the four day 16 °C treatment. Both *B. manjavacas* Russian and Petta show significant persistent enhancement of survival until day 13 (Fig. 9). *B. plicatilis* (China) also demonstrates better survival after a four day 16 °C treatment, but the magnitude of the effect is much smaller. The response of *B. rotundiformis* (Hawaii) is much different, with the 22 °C incubated animals surviving significantly better than those at 16 °C. This difference is not surprising, as *B. rotundiformis* is a species adapted to warmer temperatures and higher salinities than *B. plicatilis* (Serra et al., 1998). These differences in ecological specialization and temperature adaptation between closely related species provide insights into the differential response of rotifer strains to low temperature treatments.

Further exploration into the effects of a four day cold treatment on enhanced survival indicated that there was no benefit of reducing temperatures lower than 16 °C (Fig. 5). A four day exposure to 5 °C actually resulted in much poorer survival in early age classes. The rotifers exposed to 5 °C were also much smaller and many had algae present in their pseudocoeloms. This suggests disruption of the membrane integrity of the gut, allowing algae to leak into the pseudocoelom. This is consistent with the finding that heat and cold stress caused a decrease in membrane stability in mussels (Yao and Somero, 2012).

Enhanced survival from a four day 16 °C treatment can be further extended by subsequent intermittent exposure to 16 °C (Fig. 4). Lifespan was extended in treatments where every four days, the rotifers were transferred from 16 °C to 22 °C, or vice versa. However, there was no effect of the same transfers being performed every other day or every two days. This suggests that the regulation of the genes responsible for temperature mediated longevity effects require a specific amount of time to be activated.

Temperature during hatching of *B. manjavacas* is important because this is when embryological development occurs, and the rate of aging likely is determined in early life stages (Carrillo and Flouris, 2011). Rotifer resting eggs are arrested in the gastrulation stage, so the majority of development occurs during hatching (Boschetti et al., 2011). Upon hatching, rotifers are mostly eutelic and there is no further cell replication until new eggs are produced (Wallace, 2002). Exposure to 16 °C only during these early developmental stages was not enough to significantly extend lifespan, but 16 °C exposure during hatching as well as during the full life span increased longevity more than any other treatment (Fig. 2). The importance of early development is further highlighted by the fact that a four day exposure to 16 °C is enough to extend lifespan only if it occurs in first four days of life (Fig. 3). Exposure to 16 °C after 8 days of age provided no life extension. These results support the idea that the rate of aging is determined early in life.

There are several avenues of investigation into the mechanisms and effects of temperature dependent longevity that would benefit from further exploration. Heat-shock proteins (HSPs) are another family of genes that should be a high priority for study. HSPs are upregulated in *B. manjavacas* at high temperatures (40 °C), and in caloric restriction (Smith et al., 2012; Yang et al., 2014). Another area for further research is intraspecific differences in temperature response. Different genetic isolates of *B. plicatilis* respond differently to intermittent fasting (Gribble et al., 2014). Understanding the basis for the differential response of these naturally occurring strains can provide additional insight into the genetic basis of temperature sensitivity. Also, the RNAi approach taken in this study focused on targeting specific conserved genes. A broader RNAseq approach would provide a more comprehensive look at the genes that are differentially expressed at different temperatures, and could help identify novel genes involved in temperature sensitive pathways.

Recent theories challenge the traditional view of aging as an accumulation of molecular damage, and instead argue that aging is a result of actively regulated genetic pathways (Gems and Partridge, 2013; Blagosklonny, 2012). Our results further emphasize the importance of such genetic regulation. By activating the low temperature-sensing pathway in *B. manjavacas*, we observed enhanced survival even after thermodynamic reductions in metabolism were withdrawn. Exposure to 16 °C only enhanced longevity when exposures were during early age classes, supporting theories that the rate of aging is likely determined by environmental factors during early development (Carrillo and Flouris, 2011). Furthermore, our findings support the idea that many species harbor a latent potential for increased longevity, and that potential can be realized by environmental manipulations (Johnson et al., 1996). The many interconnected mechanisms that are responsible for temperature mediated life extension offer numerous targets for new aging interventions.

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